PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K 9/127, A01N 63/00 — A1

(11) International Publication Number: WO 00/42988

(43) International Publication Date: 27 July 2000 (27.07.00)

(21) International Application Number: PCT/US00/01277

(22) International Filing Date: 20 January 2000 (20.01.00)

(30) Priority Data:
60/116,793 21 January 1999 (21.01.99) US

(71) Applicant: UNIVERSITY OF VIRGINIA [US/US]; 1224 West Main Street, #1–110, Charlottesville, VA 22903 (US).

(72) Inventors: LINDNER, Jonathan; University of Virginia School of Medicine, Cardiovascular Division, Box 158, Charlottesville, VA 22908 (US). COGGINS, Matthew; University of Virginia School of Medicine, Cardiovascular Division, Box 158, Charlottesville, VA 22908 (US). LEY, Klaus; University of Virginia School of Medicine, Department of Biomedical Engineering, Stacy Hall 1468, Charlottesville, VA 22908 (US). KAUL, Sanjiv; University of Virginia School of Medicine, Cardiovascular Division, Box 158, Charlottesville, VA 22908 (US).

(74) Agents: REPPER, George, R. et al.; Rothwell, Figg, Ernst & Kurz, Suite 701 East, 555 13th Street N.W., Columbia Square, Washington, DC 20004 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHOD OF IDENTIFYING AND TREATING INFLAMED TISSUE

(57) Abstract

A method of identifying and/or treating tissue having leukocytes adhered thereto which utilizes a material that selectively attaches to leukocytes adhered to a patient's tissue. The material may be a gas–filled microbubble contrast agent which selectively attaches to activated leukocytes present in inflamed tissue. The microbubbles attached to activated leukocytes may be located by ultrasound echography, and inflamed tissue may be treated by a drug or gene sequence carried by the microbubble contrast agent.

METHOD OF IDENTIFYING AND TREATING INFLAMED TISSUE

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to the field of identifying and treating inflamed tissue.

DESCRIPTION OF THE BACKGROUND ART

Physicians and physiologists have long recognized the significance of local perfusion in the assessment and treatment of wounds, inflammatory disorders, viability of transplanted organs and reattached limbs, the diagnosis and treatment of cancerous lesions and the assessment and treatment of disorders of organs. Current methods for the diagnosis and treatment of these disorders are expensive, complicated and invasive procedures. It remains clear that fundamental changes in tissue perfusion are involved in disease progression and, in some cases, the onset of the diseased state.

Cancer currently accounts for 15.8% of the deaths within the United States. Methods for diagnosis are often invasive as in tissue biopsies and are often only as accurate as the technician is experienced with the specific type of cancer. Treatment ranges from radiation and chemotherapy to a variety of experimental therapies. Many of these therapies have common elements in that they are not site-specific and can have an effect on tissues not targeted.

Cardiovascular diseases are the number one cause of death in developed countries and they cause approximately 38% and 42% of deaths in Canada and the U.S. respectively. According to a 1993 American Heart Association (AHA) estimate, over 60 million Americans have one or more forms of cardiovascular disease. Each year, cardiovascular disease is responsible for approximately one million fatalities and almost 5 million individuals afflicted with the disease are hospitalized. In 1989, the AHA estimated the cost of treating cardiovascular diseases to be $88.2 billion annually.

Inflammatory diseases represent a significant concern within the human and animal health fields and considerable investments of money and research time has been spent to fight the numerous disorders falling under this classification. Inflammatory diseases can be auto-immune related, infectious or non-infectious. They can effect almost every organ and tissue type within the body. They can be common, e.g., arthritis affects up to 60% of the adult

population, or rare as in lupus which is considered an orphan disease. Diseases such as arthritis, inflammatory bowel syndrome, inflammatory liver syndrome, keratitis, lupus, ocular inflammatory disease, restinosis, psoriasis and other inflammations related to auto-immune disorders, gout, Paget's disease and lime disease represent examples of the variety of inflammatory disorders that afflict humans and animals.

As an example, pelvic inflammatory disease is a condition that is common among sufferers of sexually transmitted diseases such as chlamydia or gonorrhea. Millions of new cases are identified each year and experts predict that millions more are undiagnosed and untreated. Diagnostic procedures may be invasive and painful; many women are reluctant to undergo such testing.

A second example, inflammatory bowel disease (IBD) is a group of chronic disorders that cause inflammation or ulceration in the small and large intestines. Most often IBD is classified as ulcerative colitis or Crohn's disease but may be referred to as colitis, enteritis, ileitis, and proctitis. Ulcerative colitis causes ulceration and inflammation of the inner lining of the colon and rectum, while Crohn's disease is an inflammation that extends into the deeper layers of the intestinal wall. Ulcerative colitis and Crohn's disease cause similar symptoms that often resemble other conditions such as irritable bowel syndrome (spastic colitis). The correct diagnosis may take some time and frequently requires invasive techniques such as colonoscopy.

Physicians and researchers of the pharmaceutical industry are looking for new and effective ways of targeting drugs and genes to sites of inflammation, to areas of tissue damage and to cancerous lesions. Many delivery schemes have been developed, primarily through the formulation and coating of the drug with chemical components that modify release profiles, increase the half-life and improve general pharmacokinetic profiles. Pharmacologists have also developed numerous vehicles for delivering therapeutics into the patients, including injectable solutions, nasal sprays, oral medications, and transdermal systems, among others. Most recently, several biochemical schemes have been developed to target the drug or gene to the disease site. These schemes include linkage of antibodies or antibody fragments with the therapeutic compound, use of liposomes as delivery vehicles, and coating with specific sugars that only degrade under bacterial action. Although significant investments of capital and manpower have been made to create new delivery systems, the majority of therapeutic compounds continue to be delivered using standard techniques.

There remains a need in the art for improved methods of identifying and treating inflamed tissue.

SUMMARY OF THE INVENTION

In accordance with the present invention, a method of identifying and/or treating tissue having leukocytes adhered thereto comprises providing a material that selectively attaches to leukocytes adhered to a patient's tissue, and contacting said adhered leukocytes with said material, so as to attach said material to said leukocytes.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for identifying and/or treating inflamed tissue in a patient. Inflamed tissue typically has activated leukocytes adhered to vascular endothelium within the inflamed tissue. Suitable sites of inflammation in a patient's body include wounds, cancer, and the sites of various inflammatory diseases identified above.

It has been discovered that materials such as gas-filled microbubble shells utilized as ultrasound contrast agents when injected into a patient, attach to activated leukocytes adhered to vascular endothelium in inflamed tissue. Suitable microbubble shells can be formed of protein such as albumin and the like, phospholipid, etc. The microbubble shells can be filled with any suitable gas, such as air, perfluorocarbon gases and the like.

In accordance with one embodiment, inflamed tissue having leukocytes adhered thereto can be identified by introducing ultrasound microbubble shell contrast agent material into a patient's body having tissue not known to have leukocytes adhered thereto. Because the gas-filled microbubble shells attach to activated leukocytes present in inflamed tissue, the site of inflammation can be identified using any suitable ultrasound imaging device. The thus-identified tissue then can be treated utilizing a suitable therapeutic agent.

Because the ultrasound microbubble shells attached to activated leukocytes, tissue having activated leukocytes adhered thereto is identified by the degree of persistence of the gas-filled shells in the inflamed tissue as measured by ultrasound echography utilizing any suitable ultrasound imaging equipment. Accordingly, the amount of inflammation can be quantified based on the degree of persistence of the gas-filled ultrasound microbubble shell contrast agent in the inflamed tissue.

In accordance with one embodiment, the gas-filled microbubble shell is utilized as a carrier material for carrying the therapeutic agent to the inflamed tissue. In accordance with this embodiment, the therapeutic agent may be carried in the shell or in the gas. The activated

leukocytes adhered to the tissue are contacted with the microbubble carrier material and the agent, so as to attach the microbubble material to the leukocytes and thereby treat the tissue with the therapeutic agent. The therapeutic agent can be a medicament or insertable gene sequence for delivery to the site of inflammation.

In accordance with one embodiment, after the gas-filled shells are injected into the patient and attached to the leukocytes, the shells are bombarded with sufficient ultrasound energy to rupture the shells, and thereby facilitate contacting the tissue with the therapeutic agent.

Microbubbles can be destroyed by ultrasound energy at certain frequencies and acoustic powers normally used for ultrasound imaging. This property has been utilized to enhance the microbubble signal relative to the background tissue signal. the ability to focally destroy microbubbles with an ultrasound beam and adhesion of microbubbles to activated tissue provides method to deliver drugs (which can be packaged inside the bubble or within the shell) to areas of inflammation. This allows very high local concentrations of drugs without high systemic concentrations, thereby improving drug efficacy without added adverse effects. Moreover, since phospholipid membranes can be used as a vector for gene delivery, this can be utilized for the application of microbubble-leukocyte interactions combined with ultrasound imaging to site-specifically deliver genes to regions of inflammation.

In order to determine the mechanism of microbubble persistence in the microcirculation in the setting of endothelial injury, we directly assessed microbubble behavior within a perfused muscle by performing intravital microscopy following intravenous administration of fluorescent-labeled microbubbles. Perfluorocarbon microbubbles with either an albumin or phospholipid shell were studied within the cremaster muscle of wild-type mice at baseline, following ischemia-reperfusion injury of the muscle, and following inflammatory activation with TNF activated invasive ultrasound imaging to diagnose and follow inflammatory disorders. Moreover, these interactions provide a unique opportunity to specifically deliver drugs and genes to sites of inflammation since microbubbles can be ruptured by focused application of ultrasound energy at certain frequencies and energies.

In preferred embodiments, the effect of treatment on inflamed tissue in a patient can be monitored by periodically quantifying the amount of inflammation so as to determine if a reduction in inflammation of the inflamed tissue has occurred, by repeating the steps outlined above for identifying inflamed tissue in the patient.

The present invention may be utilized to identify tissue inflammation in a patient, target activated leukocytes in inflamed tissue, quantify the degree of inflammation based on persistence of microbubbles, follow progression of an inflammatory disease, follow response to treatment therapy of an inflammatory disease, deliver drugs or therapeutic gene sequences to cites of inflammation, identify areas of a patient's body not known to have inflammation, and the like.

Intravascular administration of gas-filled microbubbles which emit ultrasound energy within an acoustic field permits the evaluation of perfusion in various organs. During ultrasound imaging, the presence of microbubbles within the microcirculation results in opacification of the insonified tissue, such as the heart during myocardial contrast echocardiography. The stability of these microbubbles has been improved by using high molecular weight gases, which are less diffusible, and by encapsulation with a shell composed of albumin, phospholipids, carbohydrates, surfactant, or synthetic polymers. Improved stability and a small size permit transpulmonary passage of microbubbles, thereby making intravenous administration possible. Most microbubble agents behave similar to red blood cells and transit unimpeded through the normal microcirculation.

In the setting of endothelial injury produced by ischemia-reperfusion or cardioplegia delivery, albumin microbubbles persist in the microcirculation of the heart and produce prolonged opacification during myocardial contrast echocardiography. Without being bound to any particular theory, the persistence of albumin microbubbles may be due to a charge specific interaction between these anionic microbubbles and the vascular endothelium in regions where there is loss of the negatively-charged glycocalyx which coats the luminal surface of the endothelium.

To determine the mechanism of microbubble persistence, we performed intravital microscopy on the cremaster muscle of wild-type mice in order to directly visualize the microbubble behavior within the microcirculation in a variety of settings. Microbubbles containing perfluorocarbons with either an albumin or phospholipid shell were provided prelabeled with fluorescein by Mallinckrodt Inc. (St. Louis, MO) and injected into the mice intravenously at baseline, following ischemia-reperfusion injury of the muscle, and 2 hours following a potent inflammatory stimulus (TNF). The number of persistent microbubbles was related to the number of leukocytes that were adhered to the vessel wall.

These results imply tremendous clinical potential for albumin or phospholipid microbubbles to be used for imaging inflammatory disorders. Inflammation of any type requires the recruitment of circulating leukocytes into the surrounding tissue. The initial step of this process is the binding of activated leukocytes to the venular endothelium so that they may escape from the intravascular space. There is great interest in the mechanisms of leukocyte attachment to the endothelium, since interrupting this step may provide a means to treat inflammatory disorders. Our discovery of microbubble attachment of activated leukocytes provides a very unique opportunity to image inflammatory states in almost any organ system using ultrasound. This technique may be utilized not only for diagnostic purposes but also for following disease progression or response to therapy. This technique is advantageous due to the ease and safety of the technique, the widespread availability of ultrasound imaging systems, ability to perform bedside or portable studies, and the capability of obtaining immediate results.

This invention is thus useful for the diagnosis and treatment of inflammatory diseases and disorders, e.g., diagnosis and treatment of cancer, diagnosis and treatment of wounds and disorders of organs including cardiovascular disease; the use thereof to identify specific and potential sites of inflammation relating to these diseases; and the use thereof to deliver therapeutic molecules and/or genes to said sites. The invention can be used in either humans or animals.

EXAMPLE

ABBREVIATIONS AND ACRONYMS

DMSO = dimethyl sulfoxide

fMLP = N-formyl-methionine-leucine-phenylalanine

HPF = high power field

mAb = monoclonal antibody

PBS = phosphate buffered saline

PMA = phorbol 12-myristate 13-acetate

RBC = red blood cells

TNF = tumor necrosis factor

Background – Albumin microbubbles used during myocardial contrast echocardiography persist in the microcirculation of injured tissue. This study was performed to determine the mechanism and molecular determinants for microbubble persistence.

Methods and Results – Intravital microscopy of the microcirculation of exteriorized cremaster muscle was performed in 14 mice during intravenous injections of fluorescein-labeled microbubbles composed of albumin, PL^-, and PL^+. In 8 mice, injections were performed at baseline and following 30-90 minutes of ischemia with reperfusion. In 4 mice, injections were made 2 hours following TNF preactivation. Compared to baseline where few microbubbles (≤ 2 hpf^{-1}) adhered in the microcirculation, ischemia-reperfusion resulted in greater ($p<0.05$) adherence of albumin (9 ± 5 hpf^{-1}) and PL^- (5 ± 4 hpf^{-1}) microbubbles. TNF resulted in a much greater ($p<0.001$) adherence of albumin (39 ± 7 hpf^{-1}), PL^- (36 ± 10 hpf^{-1}), and PL^+ (29 ± 8 hpf^{-1}) microbubbles. In all instances, microbubbles were observed to attach to leukocytes adhered to the endothelium in post-capillary venules. Flow cytometry revealed that albumin microbubble adherence to activated leukocytes could be blocked by mAb against leukocyte integrins $\alpha_m\beta_2$ (MAC-1) and $\alpha_4\beta_1$ (VLA-4) in combination. Phospholipid microbubble adherence to activated leukocytes was partially blocked by mAb against MAC-1 and completely blocked in complement inactivated serum.

Conclusions – Microvascular persistence of microbubbles in the setting of inflammation is due to their attachment to activated leukocytes adherent to venular endothelium. Microbubble-leukocyte interactions are mediated by integrins and serum complement for albumin and phospholipid microbubbles, respectively. These results have important implications for diagnostic ultrasound imaging of inflammatory conditions and for novel methods of drug delivery.

In a normal heart, the microvascular rheology of sonicated albumin microbubbles used to assess myocardial perfusion during myocardial contrast echocardiography is similar to RBC. We have previously shown, however, that the microcirculatory transit of sonicated albumin microbubbles is abnormally prolonged in myocardial regions undergoing ischemia and reperfusion. The exact mechanism for persistence of albumin microbubbles in injured tissue is not known but has been postulated to be due to either charge-specific interactions with the microvasculature where the endothelial glycocalyx is compromised, or direct adherence to inflammatory endothelial extracellular matrix.

In the present study, intravital microscopy was used to elucidate the mechanism of prolonged microbubble transit following ischemia-reperfusion. Based on preliminary observations, it appears that both albumin and phospholipid microbubbles bind directly to activated leukocytes which are adherent to the venular endothelial surface in the setting of microvascular injury or inflammation. It further appears that microbubble-leukocyte interactions

are mediated by serum complement proteins and/or leukocyte integrins since these factors are recognized to mediate leukocyte interactions with liposomes and denatured albumin, which form the shells of several commercially available microbubble agents. This hypothesis was tested by *in vitro* assessment of the determinants of leukocyte-microbubble adhesion.

METHODS

Microbubbles and Antibodies

Perfluorocarbon-filled microbubbles (Mallinckrodt Inc., St. Louis, MO) with shells comprised of either negatively-charged denatured albumin (Optison®) or phospolipids with a net negative ($MP1950^-$) or positive ($MP1950^+$) charge were used in this study. Fluorescent labeling of the microbubbles was achieved by an amide . The mean size for the albumin, $MP1950^-$, and $MP1950^+$ microbubbles was 3.9, 5.4, and 4.3 μm, respectively. The concentration of each agent was measured prior to every experiment using a hemocytometer (Fisher Scientific, Pittsburgh, PA) and ranged between 1.8 and $4.0\times10^8/mL^{-1}$.

Murine anti-human mAb were used for the in vitro experiments to block interactions known to occur between denatured albumin and leukocyte integrins Mac-1 ($\alpha_m\beta_2$) and VLA-4 ($\alpha_4\beta_1$). These mAb included 2LPM19c (Dako Corp., Carpenteria, CA), an IgG1 mAb against the human CD11b component of Mac-1, and P4G9 (Dako Corp.), an IgG3 mAb against the human CD49d component of VLA-4. A non-specific murine IgG1 antibody (Biodesign International, Kennebunk, ME) with no known cross reactivity with human cells was used for control purposes. Flow cytometry using FITC-conjugated goat anti-mouse IgG $F(ab')_2$ (Biodesign International) for secondary labeling of the murine mAb confirmed binding of 2LPM19c mostly to neutrophils and monocytes and P4G9 mostly to lymphocytes. Only trace binding of the control antibody was detected. Complement-deficient human serum (Quidell Corp., San Diego, CA), depleted of C3 by treatment with methylamine, containing 2 mM $CaCl_2$ and $MgCl_2$ was used to assess the role of complement in microbubble-leukocyte interactions.

Animal Preparation

The study protocol was approved by the animal research committee at the University of Virginia. Twelve wild-type male C57BL/6 mice weighing between 24 and 30 g were used. Mice were anesthetized with an intraperitoneal injection (12.5 $\mu L/g^{-1}$) of a solution containing ketamine hydrocholoride (10 mg/mL^{-1}), xylazine (1 mg/mL^{-1}) and atropine (0.02 mg/mL^{-1}). Body temperature was maintained at 37°C with a heating pad. Both jugular veins were cannulated with

polyethylene tubing for administration of microbubbles and drugs. Anesthesia was maintained with intravenous administration of 0.1 mg pentobarbital approximately every 45 min as needed.

The cremaster muscle was prepared for intravital microscopy as previously described. Either the right or left cremaster muscle was exteriorized through a scrotal incision and secured to a translucent pedestal. A longitudinal incision was made in the muscle, the edges were secured to the pedestal, and the epididymis and testicle were gently pinned to the side. The cremaster muscle was superfused continuously with isothermic bicarbonate-buffered saline.

Intravital microscopy

Microscopic observations were made using an intravital microscope (Axioscop FS, Carl Zeiss, Inc., Thornwood, NY) with a saline immersion objective (SW 40/0.75 numerical aperture). Epifluorescence was performed using an excitation filter for fluorescein (450-490 nm) with a light source interfaced with a strobe (model 11360, Chadwick-Helmuth, El Monte, CA) flashing at 30 Hz. Video recordings were made using a high resolution camera (VE-1000CD, Dage-MTI, Michigan City, IN) connected to a S-VHS recorder (Panasonic, Matsushita Electric Co., Secaucus, NJ). Centerline venular RBC velocities were measured using a dual photodiode and converted to mean blood flow velocities by multiplying by an empirical factor of 0.625. Shear rates (γ_w) were determined by the equation:

$$(\gamma_w) = 2.12(8V_b)d^{-1}$$

where V_b is the mean blood velocity, d is the vessel diameter, and 2.12 is a median empirical correction factor.

Venular diameters were measured off-line using video calipers. Freeze-frame advancing allowed tracking of individual rolling leukocytes over a distance of 30 to 100 μm. The total distance traveled was divided by the elapsed time to derive the mean rolling velocity. The number of rolling leukocytes was determined by counting leukocytes crossing a line perpendicular to the vessel for 1 min. Leukocyte rolling flux fraction (F), which reflects the percent of leukocytes passing through a venule that are rolling, was calculated by:

$$F = r_n(0.25\pi d^2/V_b/60/C_L)^{-1}$$

where r_n is number of rolling leukocytes in 1 minute, d is vessel diameter, V_b is centerline blood velocity, and C_L is the systemic leukocyte blood concentration. Adherent leukocytes, defined as those not moving for at least 30 sec, were counted and expressed per surface area of the venule, calculated from diameter and length assuming cylindrical geometry of the venule.

***In vivo* Protocols**

Cremaster muscles from 8 wild-type mice were used to assess the microbubble behavior following ischemia and reperfusion injury. Prior to ischemia, 3 venules with diameters between 25 and 40 μm were recorded under transillumination for 1 min each followed by measurement of centerline blood velocity. Intravenous injections of each microbubble agent were performed in random order. Approximately $4.0x10^7$ microbubbles (volume range 100 - 220 μL) were injected over 20s. Two minutes following each injection, 50 random hpf encompassing arterioles, venules, and capillaries were scanned over approximately 2 minutes using fluorescent epi-illumination. Brief transillumination was used to confirm the presence of normal flow in a vessel when adherent microbubbles were identified. Blood flow was then interrupted for 30 to 90 min using a ligature placed around the cremasteric artery and, if present, around the major feeding artery connecting the cremaster to the epididymis. The microcirculation was monitored to ensure cessation of flow over the entire ischemic period. Following 20 min of reflow, microbubble administration was again performed in random order. The three venular segments recorded prior to ischemia were then identified by anatomic landmarks for post-reperfusion video recordings and velocity measurements.

Four wild-type mice were studied to assess microbubble behavior in a model of inflammation induced by TNF-α activation. Intrascrotal injections of 0.5 μg murine recombinant TNF-α (Genzyme Corp., Cambridge, MA) in 0.2 mL sterile saline were performed 2 hours prior to dissection of the cremaster muscle. Video recordings and velocity measurements of 3 venules and microbubble injections were performed in a manner similar to the ischemia-reperfusion protocol.

Flow cytometry

Blood was obtained from healthy adult volunteers, anticoagulated with heparin (10 U/mL^{-1}), and centrifuged to separate the cellular and serum components. The cellular fraction was washed and leukocytes were fluorescently labeled by the addition of rhodamine-6-G (Molecular Probes Inc., Eugene, OR) to a final concentration of 1 μM for 30 min. Cells were then washed twice, resuspended in PBS, and analyzed for leukocyte concentration using hemocytometer measurements of Kimura-stained samples. Leukocytes were activated by 10 nM PMA (Sigma Chemical Co., St. Louis, MO) dissolved in DMSO (Sigma Chemical Co.) for 15 min at 37°C. Serum fractions from the normal volunteers and complement deficient serum were kept on ice until just prior to use.

Approximately $2x10^6$ leukocytes in 0.2 mL of PBS containing 2 mM $MgCl_2$ were incubated at 37°C for 3 minutes with $2x10^7$ albumin, MP1950$^-$, or MP1950$^+$ microbubbles in the presence of either serum, complement-deficient serum, or PBS containing 2 mM $MgCL_2$ to a total volume of 0.4 mL. To some of these samples, 10 μg of IgG_1 isotype control antibody and mAbs 2LPM19c and P4G9, alone and in combination, were added prior to the addition of microbubbles. Red blood cells were hypotonically lysed and samples were analyzed using a laser flow cytometer (FACScan, Beckton-Dickson, San Jose, CA). Differences between leukocyte and microbubble side and forward light scatter permitted exclusion (by live gating) of free microbubbles from analysis. Data are presented as green (fluorescein) versus red (rhodamine) fluorescence and as histograms of green fluorescence in a gated population.

Statistical Analysis

Data are expressed as mean±SD. Comparisons were made by 1-way repeated-measures ANOVA. Correlations between leukocyte rolling or adherence and microbubble persistence were made by multiple regression analysis. Differences were considered significant at $p<0.05$ (2-sided).

RESULTS

In vivo experiments

Two of the 8 mice undergoing cremasteric ischemia died during arterial occlusion, precluding reflow data in these animals. The duration of ischemia in the remaining mice was 30, 60 and 90 min (2 mice each). Venular hemodynamic data were taken from all mice. In mice undergoing ischemia-reperfusion, mean blood velocity and shear rate were slightly higher following reflow compared to baseline, probably as a result of hyperemia, but these differences did not reach statistical significance. Leukocyte rolling in post-capillary venules was observed prior to ischemia. The mean rolling velocity (36.9 ± 19.3 $\mu m/s^{-1}$) and flux fraction (0.19±0.06) was consistent with those previously measured early after exteriorization of the cremaster and is mediated by P-selectin expression following surgical trauma. Following ischemia and reperfusion, the leukocyte rolling velocity and flux fraction decreased, although the change in the latter did not reach statistical significance. The mean number of adherent leukocytes almost doubled following ischemia and reperfusion. There was no relation between the duration of ischemia and either rolling flux fraction or number of adherent leukocytes and all reperfusion data was therefore pooled.

Compared to ischemia-reperfusion injury, TNF-α resulted in markedly slower mean leukocyte rolling velocity, despite only mild differences in shear rate. This observation is consistent with E-selectin and integrin-mediated slow rolling following cytokine-induced inflammation. TNF-α resulted in a very high number of adhered leukocytes which, together with leukocyte transmigration, were probably responsible for the low calculated leukocyte flux fraction.

In animals undergoing cremasteric ischemia, microbubble adherence was uncommon at baseline. Following ischemia and reperfusion, adherence of albumin and MP1950$^-$ microbubbles was observed in venules only. Following TNF-α, there was much greater venular attachment for all 3 microbubble preparations. In almost all instances, microbubbles appeared to attach to the surface of leukocytes adhered to the endothelial surface of venules. Venules from 2 different mice were observed following ischemia-reperfusion (panel A) and following TNF-α activation (panel B). Images obtained by transillumation (left panels) demonstrated a greater degree of leukocyte adherence following TNF-α since most of the leukocytes in panel A were rolling and about 60% in panel B were adhered. Fluorescent epi-illumination (right panels) of the same venular segments following injection of albumin microbubbles revealed microbubbles attached to individual adhered leukocytes (A) and clusters of leukocytes (B) adhered to the venular surface. Occasionally, adherent leukocytes coupled with microbubbles were observed to release and either roll for a short distance and adhere in a new location or join streamline flow, conveying the microbubble with them. All microbubbles observed rolling along the endothelial surface appeared to be physically associated with a slowly rolling leukocyte and constituted only 6% of all microbubbles interacting with leukocytes. The degree of leukocyte adherence correlated with the venular persistence of albumin ($r=0.72$, $p=0.004$), MP1950$^-$ ($r=0.73$, $p=0.01$), and MP1950$^+$ ($r=0.53$, $p=0.03$) microbubbles. No correlation was found between the leukocyte rolling flux fraction and microbubbles adhered.

Potential mechanisms of interactions between leukocytes and microbubbles were evaluated using flow cytometry. Activated leukocytes (A) labeled with Rhodamine-6G exhibited high activity in the red spectrum but with little overlap into the green spectrum. The appearance of events in the upper right quadrant (combined red and green fluorescence) when cells were combined with the different preparations of fluorescein-labeled microbubbles in the presence of serum indicated interactions between the two. Recorded events were gated according to forward and side scatter to exclude the smaller free microbubbles and variability in the extent of green fluorescence probably represented variation in both the number of microbubbles attached and

microbubble size. The fraction of leukocytes with microbubbles attached for albumin, MP1950$^-$, and MP1950$^+$ microbubbles 50%, 51%, and 32%, respectively. Inhibition of albumin microbubble attachment to leukocytes by blocking Mac-1 was observed by histograms of green fluorescent activity. PMA-activated leukocytes (A) exhibited little green fluorescent activity whereas albumin microbubbles (B) were strongly fluorescent. When incubated together (C), leukocyte-microbubble complexes were evident by the appearance of fluorescence on neutrophils (free microbubbles were again excluded from this analysis by their scatter characteristics). Interactions between albumin microbubbles and neutrophils were largely blocked by the mAb against the CD11b component of Mac-1 (D). Inhibition of Mac-1 resulted in 63% reduction in albumin microbubble binding to activated leukocytes. No apparent inhibition occurred with either the control antibody or the mAb for the CD49d component of VLA-4 or when incubations were performed in PBS or complement-depleted serum. Corresponding results for MP1950$^-$ and MP1950$^+$ were obtained.

DISCUSSION

Until now, the mechanisms responsible for albumin microbubble persistence within the microcirculation following injury have been speculative. In the present study, we discovered that albumin and phospholipid microbubbles, irrespective of charge, bind to activated leukocytes that have adhered to post-capillary venules following ischemia and reperfusion injury and during inflammation induced by TNF-α. Our flow cytometery results suggest that interactions between leukocytes and albumin microbubbles are mediated, in part, by leukocyte integrins that play a major role in inflammation. Together, these findings indicate that microbubble agents traditionally used to assess perfusion in organ systems also may be utilized for the assessment of a wide variety of inflammatory disorders.

Mechanisms of Microbubble-Leukocyte Interactions

In order to define the mechanisms responsible for persistent myocardial opacification following albumin microbubble injections into injured vascular beds, we directly observed microbubble behavior in the microcirculation of the mouse cremaster muscle. Under normal conditions, microbubbles were observed to pass through the microcirculation unimpeded. Following either ischemia-reperfusion or TNF-α activation, albumin and phospholipid microbubbles persisted in post-capillary venules by means of their binding to activated leukocytes on the endothelial surface. Direct interactions between microbubbles and injured microvascular endothelium was rarely observed. The methods used to produce inflammation in this study result in a significant heterogeneity in the extent of leukocyte adhesion between vessels and, by

observing 50 HPF, we were able to confirm that microbubble adherence preferentially occurred in venular segments with the greatest leukocyte accumulation and did not occur in regions devoid of leukocytes.

Our hypothesis that microbubbles adhere to activated leukocytes by means of leukocyte adhesion molecules was based, in part, on observations made during intravital microscopy. In this and other studies, trauma incurred during exteriorization of the cremaster muscle resulted in leukocyte rolling in venules. This leukocyte rolling results from high dissociation rate bonds between endothelial P-selectin, which is rapidly translocated to the surface from Weibel-Palade bodies, and its glycoprotein ligand PSGL-1 on the leukocyte surface. Despite a mean rolling flux fraction of 0.19 prior to ischemia, attachment of microbubbles to rolling leukocytes at baseline was rare. The phenomenon of leukocyte rolling, in itself, does not denote leukocyte activation but, instead, represents an initial step of the inflammatory cascade whereby capture of leukocytes facilitates both time-dependent activation by local chemokines and subsequent development of higher affinity bonds with low forward reaction rates. The higher affinity bonds that result in arrest of leukocytes (and transmigration) are mediated by leukocyte integrins which, when activated, interact with immunoglobulin receptors (ICAM-1, VCAM-1) on the endothelial surface. Firm leukocyte adherence at baseline caused by surgical trauma in this study was present only to a small degree and is normally not observed until greater than 1 hour after exteriorization of the muscle. Leukocyte adhesion within venules was more pronounced following ischemia-reperfusion and TNF-α activation, especially with the latter. It was this population of cells that interacted with microbubbles. As a result, the extent of microbubble persistence in the microcirculation correlated with adhesion density but not rolling flux fraction.

The concept that albumin microbubbles preferentially adhere to activated leukocytes is also supported by non-specific interactions known to occur between leukocyte integrins and a wide variety of proteins. β_2 integrins, which play a critical role in neutrophil and monocyte adhesion and extravascular transmigration, bind to a diverse range of protein substrates other than endothelial immunoglobulin adhesion molecules, including fibrinogen, fibronectin, vitronectin, laminin, casein, and albumin. Isolated human leukocytes and monocyte-differentiated HL-60 cells have been shown to bind to albumin following their activation with PMA or fMLP. These interactions are almost entirely inhibited by monoclonal antibody blockade of the CD18 subunit of the β_2 integrins and, more specifically, by antibodies specific for Mac-1 (CD11b/CD18). Mac-1-mediated adhesion is much greater with denatured rather than native albumin, promoting the

concept that neutrophils may be directed to regions of abnormal extracellular matrix and retained in injured tissue by means of β_2 integrin binding to denatured proteins. Similarly, denatured albumin has been shown to be a chemotactic factor for neutrophils. In the current study, *in vitro* interactions between activated leukocytes and albumin microbubbles, the shells of which contain mostly albumin that has been denatured by thermal energy during sonication, were inhibited by 63% by an antibody against Mac-1. Accordingly, ischemia-reperfusion and TNF-α, both of which result in an inflammatory response characterized by β_2 integrin-dependent leukocyte adhesion, resulted in microbubble binding to leukocytes.

Clinical relevance of microbubble-leukocyte interactions

Persistent myocardial opacification has been observed during myocardial contrast echocardiography with albumin microbubbles in the setting of microvascular injury produced by ischemia-reperfusion, crystalloid cardioplegic arrest, and radio frequency catheter ablation. In these studies, the microbubble appearance (wash-in) rates, derived from time-intensity data, were essentially normal whereas the decay (washout) rates were prolonged. These findings are consistent with our observations that microbubbles attach to leukocytes which adhere to post-capillary venules and not to arterioles or capillaries. The initial studies of cardiac ischemia demonstrated a relation between ischemic duration (up to 45 min) and adherence of albumin microbubbles during reperfusion. This finding is consistent with influence of ischemic time on leukocyte adherence in different tissues/organ systems which appear to be dependent upon the extent of Mac-1 activation. The absence of a relation between ischemic time and either leukocyte adherence or microbubble persistence in the current study may be attributable to relatively longer durations of ischemia.

Regardless of whether microbubbles attach to specific leukocyte adhesion molecules that are activated and play a major role in leukocyte adhesion during inflammation or bind in a non-specific or indirect (complement-mediated) fashion, the extent of binding appears to reflect the extent of leukocyte adherence very early in the inflammatory cascade.

The ability to quantify the degree of microvascular inflammation in a variety of organ systems and disease states using a non-invasive technique such as contrast-enhanced ultrasound may provide a very attractive alternative to many current techniques that are invasive (biopsy) or indirect (serologic markers). The safety and efficacy of many different microbubble contrast agents has already been established.

CLAIMS

1. A method of treating tissue having leukocytes adhered thereto, comprising

- providing a material that selectively attaches to leukocytes adhered to a patient's tissue, said material being a carrier material carrying an agent for treating said tissue; and

- contacting said adhered leukocytes with said carrier material and said agent, so as to attach said carrier material to said leukocytes and treat said tissue with said agent.

2. The method of claim 1 wherein said leukocytes adhered to said tissue are activated leukocytes.

3. The method of claim 1, wherein said carrier material is comprised of gas-filled microbubble shells.

4. The method of claim 3 wherein said agent is carried in said shell or in said gas.

5. The method of claim 3 wherein said shell is comprised of protein or phospholipid.

6. The method of claim 3 wherein said shell is comprised of albumin.

7. The method of claim 1 wherein said tissue is inflamed tissue.

8. The method of claim 7 wherein said leukocytes are adhered to vascular endothelium in said inflamed tissue.

9. The method of claim 3 wherein said gas-filled shells comprise an ultrasound contrast agent when injected into the patient.

10. The method of claim 9 wherein, after said gas-filled shells are attached to said leukocytes, said shells are bombarded with sufficient ultrasound energy to rupture said shells, so as to contact said tissue with said agent.

11. A method of identifying and treating tissue having leukocytes adhered thereto, comprising:

A) providing a material that selectively attaches to leukocytes adhered to tissue in a patient's body;

B) introducing said material into an area of the patient's body having tissue not known to have leukocytes adhered thereto;

C) identifying tissue in the patient's body having leukocytes adhered thereto by locating where said material is attached to the adhered leukocytes;

D) treating said tissue with a therapeutic agent.

12. The method of claim 10 wherein said tissue is inflamed tissue.

13. The method of claim 10 wherein said leukocytes are activated leukocytes.

14. The method of claim 11 wherein said leukocytes are adhered to vascular endothelium in said inflamed tissue.

15. The method of claim 10 wherein said material is comprised of gas-filled microbubble shells.

16. The method of claim 14 wherein said shells are comprised of protein or phospholipid.

17. The method of claim 14 wherein said shells are comprised of albumin.

18. The method of claim 14 wherein said gas-filled shells comprise an ultrasound contrast agent when injected into said patients body.

19. The method of claim 14 wherein said tissue having leukocytes adhered thereto is identified by a degree of persistence of the gas-filled shells in the inflamed tissue as measured by ultrasound echography.

20. The method of claim 18, further comprising the step of quantifying an amount of inflammation of said tissue based on the degree of persistence of said gas-filled shells in said inflamed tissue.

21. The method of claim 19, further comprising repeating steps (A) through (C), and further quantifying said amount of inflammation, so as to determine if a reduction in inflammation of said inflamed tissue has occurred.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/01277

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 9/127; A01N 63/00
US CL : 424/450, 93.71
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450, 93.71

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ROVAI, D, et al. Residual Myocardial Perfusion in Reversibly Damaged Myocardium by Dipyridamole Contrast Echocardiography. European Heart Journal. 1996, Vol. 17, pages 296-301, see entire document.	1-21
Y	VILLANUEVA, F.S. et al. Albumin Microtubule Adherence to Human Coronary Endothelium: Implications for Assessment of Endothelial Function Using Myocardial Contrast Echocardiography. Journal of the American College of Cardiology. September 1997, Vol. 30, No. 3, pages 689-693, see entire document.	1-21
Y	BROWN, J.M. et al. Contrast Enhanced Sonography of Visceral Perfusion Defects in Dogs. Journal of Ultrasound Medicine. 1997, Vol. 16, pages 493-499, see entire document.	1-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
	19 APR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No.	Authorized officer JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX Telephone No.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/01277

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DALECKI, D. et al. The Influence of Contrast Agents on Hemorrhage Produced by Lithotripter Fields. Ultrasound in Medicine and Biology. 1997, Vol. 23, No. 9, pages 1435-1439, see entire document.	1-21

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 504508060WO0	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US04/31291	International filing date (*day/month/year*) 22 September 2004 (22.09.2004)	(Earliest) Priority Date (*day/month/year*) 22 September 2003 (22.09.2003)
Applicant AVI BIOPHARMA, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 2 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the Report**
   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
      ☐ The international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.
2. ☐ **Certain claims were found unsearchable** (See Box No. II)
3. ☐ **Unity of invention is lacking** (See Box No. III)
4. With regard to the **title**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,
   a. the figure of the **drawings** to be published with the abstract is Figure No. ____
      ☐ as suggested by the applicant.
      ☐ as selected by this Authority, because the applicant failed to suggest a figure.
      ☐ as selected by this Authority, because this figure better characterizes the invention.
   b. ☐ none of the figures is to be published with the abstract.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US04/31291

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 9/127
US CL : 424/450
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/450

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST BRS SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,498,421 A (GRINSTAFF et al.) 12 March 1996 (12.03.1996), see abstract; column 7, line 61 - column 32, line 9.	1-4, 6-22, 24-26
X --- Y	US 2001/0051131 A1 (UNGER) 13 December 2001 (13.12.2001), see paragraphs 0079, 0085, 0087, 0190, 0295, 0300.	1-5, 9, 11-13, 17-21, 23 --- 6-8, 12-16, 22, 24-26

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
27 November 2004 (27.11.2004)

Date of mailing of the international search report
01 DEC 2004

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
Facsimile No. (703) 305-3230

Authorized officer
Micah-Paul Young

Telephone No. (571) 272-1600

Form PCT/ISA/210 (second sheet) (January 2004)